**Sudan University of Science and Technology**

**College of Graduate Studies**

# Evaluation of Plasma Level Interleukin-12 among Sudanese Hepatitis B Patients in Khartoum State.

# تقييم مستوى المادة الخلوية 12 البلازمية وسط المرضي السودانيين بالتهاب الكبد الوبائي النوع (ب) بولاية الخرطوم.

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# الأية

**بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيم**

(وَوَصَّيْنَا الْإِنْسَانَ بِوَالِدَيْهِ إِحْسَانًا حَمَلَتْهُ أُمُّهُ كُرْهًا وَوَضَعَتْهُ كُرْهًا وَحَمْلُهُ وَفِصَالُهُ ثَلَاثُونَ شَهْرًا حَتَّى إِذَا بَلَغَ أَشُدَّهُ وَبَلَغَ أَرْبَعِينَ سَنَةً قَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَى وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَصْلِحْ لِي فِي ذُرِّيَّتِي إِنِّي تُبْتُ إِلَيْكَ وَإِنِّي مِنَ الْمُسْلِمِين)

صدق الله العظيم

سورة الاحقاف (15)

# Dedication

**To**

My father and mother with all love and respect ..

My great supervisor ..

My husband and my little baby ..

My brother and my sister ..

My best friends ..

## Acknowledgement

First of all I thank great full to ALMOGITH ALLAH who give me ability and strongly to complete this research.

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# Abstract

Hepatitis B virus remains a major global pathogen that cause acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, Interleukin-12(IL 12) cytokine has ability to induce interferon gamma, probably plays an important role in the antiviral immune response to HBV during natural infection. This is case-control study was aimed to evaluate plasma Interleukin-12(IL-12) level in Sudanese patients infected with Hepatitis B virus(HBV) compared to healthy control group in Saba laboratory (hepatitis B virus infections center) in Khartoum state during the period from April to February (2020). Eighty subjects, selected randomly in this study, with age varies from 12-70 years. Fourty subjects are hepatitis B patients as case group with mean of age 30.2±12.3 years and fourty subjects as aberrantly healthy group with mean of age 34.3±12.2 years are age and sex matched healthy control group. Three milliliter of Venous blood sample were collected in EDTA container from each subjects and separated using centrifuge to obtain plasma. IL-12 concentration was measured by Enzyme Linked Immunosorbent Assay (ELISA) in Ibn Sina University Research Center. The data was analyzed using statistical package of social science programmed (Version 20). students' T test and one-way ANOVA test were used to compare between means. P-value significant when ≤0.05. Mean level of IL-12 was significantly increased in HBV patients (693.94± 898.9pg/ml) compared to control group (181.18±124.3 pg/ml) with (p-value 0.001). Mean level of IL-12 in hepatitis B patients was lower in males than female which is statistically significant (P-value 0.02). There was statistical significant correlation between IL-12 level and treated, vaccinated patients and patients receive blood transfusion (p-value 0.05, 0.036, 0.025) respectively .IL-12 level did not affected with age group, symptomatic and asymptomatic patients among case subject p.value showed insignificant correlation (P-value 0.5, 0.282) respectively .This study concluded that IL-12 concentration could be a useful prognostic marker for HBV infection.

# مستلخص البحث

يظل فيروس الكبد البائي النوع ب احد العوامل المسببة للامراض العالمية التي تسبب التهاب الكبد الحاد والمزمن, تليف الكبد وسرطان الكبد. المادة الخلوية 12 من المواد الخلوية التي لها القدرة علي حث غاما انترفيرون وربما تلعب دورا هاما في الاستجابة المناعية للفيروسات الكبد البائي خلال العدوي الطبيعية.هذة دراسة الحالة والضابطة هدفت الي تقيم مستوي المادة الخلوية 12 لدي المرضى السودانين المصابين بالتهاب الكبد ب الفيروسي مقارنة مع الافراد الطبيعين بولاية الخرطوم في الفترة من ابريل الي فبراير 2020 . اختير ثمانون فردا" عشوائيا" لهذه الدراسة وكانت أعمارهم تتراوح من 12الي 70 عاما,"40 مريضا من مرضى التهاب الكبد ب الفيروسي متوسط اعمارهم 30.2±12.3 عاما" و40 من الافراد الطبيعين متوسط اعمارهم 34.3±12.2 عاما متوافقين في العمر والجنس. سحبت ثلاثة مليلتر عينة دم وريدية من كل مشارك في انبوبة تحتوي علEDTA وفصلت باستخدام الطرد المركزي وحصل علي البلازما .قيس تركيز المادة الخلوية 12 عن طريق فحص الممتز المناعي المرتبط بالانزيم في مركز ابحاث جامعة ابن سينا. حللت البيانات باستخدام الحزمة الاحصائية للمجتمع (نسخة 20 ).استخدم انوفاANOVA) ) واختبار T لمقارنة الاوساط وكانت القيمة المطلقة متوافقة عند اقل من 05.0. كان هناك ارتفاع ذو دلالة احصائية للوسط الحسابي للمادة الخلوية 12 في المرضي بالتهاب الكبد ب الفيروسي 898.9±693.94 بيكوجرام\مل مقارنة بالافراد الطبيعين 181.18±124.3 بيكوجرام \مل (القيمة الاحتمالية 0.001). . متوسط المادة الخلوية 12 في مرضى التهاب الكبد ب الفيروسي اقل في الذكور من الاناث حيث يوجد فرق ذا دلالة احصائية (القيمة الاحتمالية.02 0) .كانت هناك علاقة ذات دلالة إحصائية بين مستوى المادة الخلوية 12 والمعالجين ، والمرضى الذين تم تلقيحهم والذين يتلقون نقل الدم (القيمة الأحتمالية 0.05 ، 0.036 ، 0.025 ) على التوالي.لم يتأثر مستوى المادة الخلوية 12 بالفئة العمرية ، ولم يتأثر المرضى الذين يعانون من أعراض أو أعراض بين حالة الحالة وأظهرت القيمة الاحتمالية ارتباط غير مهم (القيمة الاحتمالية 0.5 و 0.282 ) على التوالي. خلصت هذة الدراسة الي ان تركيز المادة الخلوية 12 قد يكون نذير مفيد لعدوي التهاب الكبد ب الفيروسي.

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## List of Abbreviations

| Abbreviations | full name |
|---|---|
| Ag | Antigen |
| Ab | Antibody |
| AHB | Acute Hepatitis B |
| ANOVA | Analysis Of Variance |
| ALF | Acute Liver Failure |
| ALT | Alanine Amino Transferase |
| AST | Aspartate Amino Transferase |
| CCCDNA | Covalently Closed Circular Deoxy Nucleic Acid |
| CD | Clusters Of Differentiation |
| CDC | Center For Disease Central |
| CHB | Chronic Hepatitis B |
| DC | Dendritic cell |
| DNA | Deoxy Ribonucleic Acid |
| DTaP | Diphtheria Tetanus Pertussis |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| G-CSF | Granulocyte Colony Stimulating Factor |
| HBV | Hepatitis B Virus |
| HBcAg | Core antigen of Hepatitis B |
| HBIG | Hepatitis B Immunoglobulin |
| HBsAg | Surface Antigen of Hepatitis B |
| HBxAg | X antigen of Hepatitis B |
| HCC | Hepatocellular carcinoma |
| HIV | Human Imunodiffiency Virus |
| HRP | Horseradish peroxide |

| | |
|---|---|
| IFN | Interferon |
| IL-12R-β | Interlukin-12 Receptor-Beta |
| TNF | Tumor Necrosis Factor |
| IFN-G | Interferon-Gamma |
| IgA | Immunoglobulin A |
| IgE | Immunoglobulin E |
| IgM | Immunoglobulin M |
| IgG | Immunoglobulin G |
| IV | Intravenous |
| IU/ML | International unit per milliliter |
| ILs | Interleukins |
| NIH | National Institute Of Health |
| NK | Natural killer |
| Ng/ml | Nanogram per milliliter |
| MHC | Major Histocompatibility Complex |
| MFI | Mean Fluorescence Intensity |
| PBMC | peripheral Blood Mononuclear Cell |
| PCR | Polymerase Chain Reaction |
| Pg/ml | Pictogram Per Milliliter |
| PV | Probability Value |
| RNA | Ribonucleic Acid |
| TBM | Tubular Basement Membrane |
| TGFB | Tumor Growth Factor Beta |
| Th | T helper |
| SD | Stander Deviation |
| STA | Signal Transducer and Activator of Transcription |
| SPSS | Statically Package For Social Sciences |
| TNFα | Tumor Necrosis Factor Alfa |

| | |
|---|---|
| TNFβ | Tumor Necrosis Factor Beta |
| TNFR | Tumor Necrosis Factor Receptor |
| WHO | World Health Organization |

# Chapter One

## Introduction

# CHAPTER I
# INTRODUCTION

## 1.1.Introduction

Hepatitis B virus is an important human pathogen that has caused chronic infections worldwide (Karmvis and Kew, 2010).

Recent data obtained from a modeling study has shown that the global prevalence of hepatitis B surface antigen (HBsAg) was 3.9% in 2016, corresponding to an estimated 290 million infections worldwide (Wu *et al*., 2017).

Hepatitis B virus is a common nosocomial infection that causes higher rate of mortality and morbidity in blood recipients. Sudan has a seroprevalence of greater than 8% HBsAg positivity ( Idrees, 2019 ).

Hepatitis B virus infection causes a broad spectrum of liver diseases ranging from acute to chronic hepatitis B infection with no biochemical evidence of liver injury to progressive chronic hepatitis B, which may advance to liver cirrhosis, liver failure, and hepatocellular carcinoma (Zhang *et al*.,2018 ).

Globally, more than 2 billion people have been infected with HBV at some time in their lives. About 400 million of HBV carriers in the world complications such as fulminant hepatic failure, cirrhosis and hepatocellular carcinoma develop annually in 250 000 of them ( Ozguler *et al*., 2015).

Interleukin-12 (termed IL-12p70 and commonly designated IL-12) is an important immunoregulatory cytokine that is produced mainly by antigen-presenting cells. (Kalliolias and Ivashkiv, 2016) .

Expression of IL-12 during infection regulates innate responses and determines the type of adaptive immune responses. IL-12 induces interferon-Gamma production and triggers CD4+ T cells to differentiate into type 1 T helper (Th1) cells. (Abbas *et al*., 2015).

Studies have suggested that IL-12 could play a vital role in treating many diseases, such as viral and bacterial infections and cancers . **(**Ippolito *et al*.,2010).

## 1.2 Rationale

Hepatitis B virus (HBV) is a major blood-borne and sexually transmitted infectious agent, and represents a serious global public health problem. It is approximately 100 times more contagious than human immunodeficiency virus (HIV) and is found in diverse populations and subpopulations (Yeshi *et al*., 2014 ).

In addition, several results suggest that IL-12, through its ability to induce interferon gamma, probably plays an important role in the antiviral immune response to HBV during natural infection. Further,IL-12 may have therapeutic value as an antiviral agent for the treatment of chronic HBV infection. ( Cavanaugh,2011).

There was no published data studied plasma level of IL-12 among hepatitis B patients locally ,so study was counducted.

## 1.3.Objectives

### 1.3.1.General objectives

-To evaluate plasma level of Interleukine-12 among Sudanese HBV disease patients in Khartoum State.

### 1.3.2.Specific objectives

- To measure level of IL12 in HBV disease patients and in apparently healthy volunteers using Enzyme Linked Immunosorbent Assay.
- To compare between plasma levels of IL12 in both HBV patients and normal controls .
- To correlate IL12 level with possible risk factors e.g age, gender, treatment, blood transfusion,and jaundice, vaccination .

# Chapter Two

## Literature Review

# CHAPTER II

# LITERATURE REVIEW

## 2.1. Hepatitis B morphology

### 2.1.1. structure

Hepatitis B virus,abbreviated HBV, is a partially double –stranded DNA virus ( Ryu,2017) . It is belongs to the Orthohepadna genus of Hepadnaviridae family of virus (Hunt,2011) .

The virus particle, called Dane particle (virion), consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses. The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells( Locarnini,2013).

Hepatitis B is one of the smallest enveloped animal viruses with a virion diameter of 42 nm, but pleomorphic forms exist, including filamentous and spherical bodies lacking a core. These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus (Robinson,2010).

### 2.1.2. Components

Hepatitis B virus is consists of HBsAg, HBcAg (HBeAg is a splice variant), Hepatitis B virus DNA polymerase, HBx the function of this protein is not yet well known, but evidence suggests it plays a part in the activation of the viral transcription process.(Benhenda and Ducroux,2013).

### 2.1.3. Genome

Hepatitis B is a noncytopathic enveloped virus with circular double strand DNA genome that cause acute and chronic inflammatory liver disease and hepatocellular carcinoma ( Hassan and Almazrou, 2018) .

The genome is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase, The genome is 3020–3320 nucleotides long (for the full length strand) and 1700–2800 nucleotides long (for the short length strand) (Zoulim and Mason, 2010).

### 2.1.4. Epidemiology

According to the WHO and the Centers for Disease Control and Prevention (CDC), 257 million people are living with HBV. Moreover, 20,900 acute hepatitis B cases were reported in 2016. Hepatitis B is highly prevalent in the African, Western Pacific, Eastern Mediterranean, South-East Asia, and European regions, respectively (AlSadeq ,2019).

According to a recent systematic review and meta-analysis estimating the worldwide prevalence of chronic HBV infection, in 2010, about 248 million individuals were hepatitis B surface antigen (HBs Ag) positive. More than 780,000 people die every year due to hepatitis Brelated complications including cirrhosis and hepatocellular carcinoma (Schweitzer *et al.*,2015) . Prevalence of HBV chronic infection is particularly high in Africa reaching up to 22 % in South Sudan (WHO,2016).

In Cameroon, seroprevlance of HBs Ag has been reported to be 10.1 % in a general population of blood donors, 10.2 % among pregnant women and 23.7 % among HIV-infected patients (Tatsilong ,2016).

Sudan is classified among the African countries with high HBV endemicity. The reported prevalence of HBV chronic infection, characterized by the detectable level of HBV surface antigen (HBsAg) varied from region to region and ranged between 5 and 7% in the general population and 26% in hospital outpatients ( Mahgoub,2011). Variation in the prevalence rates reflects the variation in the risk factors associated with the infection in each country . Despite the few studies on the hepatitis B situation in the Arabic world, the prevalence of chronic HBV infection was found to be decreasing in some

Arabic countries, such as Arabian Gulf, Lebanon, Egypt and Libya (Hamad *et al*., 2019).

### 2.1.5. Transmission

HBV has been found in virtually all body secretions and excretions. However, only blood, body fluids containing visible blood, semen and vaginal secretions represent a risk of transmission. HBV is transmitted by percutaneous and mucosal exposure to infective blood or body fluids (Heiberg *et al.*, 2010).

Major modes of HBV transmission include sexual or close household contact with an infected person, perinatal mother to infant transmission, injecting drug use and nosocomial exposure (Hahne *et al*., 2013). Percutaneous exposures that have resulted in HBV transmission include transfusion of unscreened blood or blood products, sharing unsterilized injection needles for IV drug use, haemodialysis, acupuncture, tattooing and injuries from contaminated sharp instruments sustained by hospital personnel (Navabakhsh *et al*., 2011).

HBV is stable on environmental surfaces for at least 7 days and is 100 times more infectious than HIV. However, after introduction of the vaccination, significant reduction in its transmission has been detected (Stasi *et al*., 2017).

### 2.1.6. Replication

HBV chronically infects hepatocytes. It replicates by reverse transcription of an RNA intermediate, the pregenome. Nuclear cccDNA formed from the incoming relaxed circular viral DNA, serves as the transcriptional template (Shuping , 2016). Progeny genomes are formed by reverse transcription, which occurs within viral nucleocapsids in the cytoplasm of infected cells. Nucleocapsids with mature viral DNA are either assembled into viral envelopes and exported from the infected hepatocyte or, if needed, transported to the nucleus to amplify cccDNA copy number. Envelpe proteins are also secreted as subviral particles, hepatitis B surface antigen (HBsAg), as are large numbers of virus-like particles with empty nucleocapsids (Hu and Seeger, 2015).

While the broad outline of infection and replication are clear, gaps in knowledge still exist at the molecular level still do not know all of the steps in disassembly of viral nucleocapsids, delivery of the viral genome into the cell nucleus, and cccDNA formation. (Hu, 2017).

cccDNA transcription is clearly dependent upon liver specific transcription factors, but the roles of virus proteins in HBV transcription are still nuclear, HBx is needed for efficient transcription from cccDNA, but the mechanism is not yet known (Zijie *et al*., 2019). While it is likely that HBx recruits a cellular protein to the Cullin4-DDB1 E3 ligase, the nature of the cellular proteins remain elusive. Packaging into nucleocapsids of pregenomic RNA and revers transcriptase are essential for viral DNA synthesis, and packaging of a variety of cellular factors, including a kinase, chaperones and members of the APOBEC family of proteins, have also been reported (Seeger and William, 2018).

### 2.1.7. Hepatitis B mediated liver diseases

The infecting dose of virus and the age of the person infected are important factor that correlate with the severity of acute or chronic hepatitis B (Barker and Murray,2010). Primary HBV infection may be associated with little or no liver disease or with acute hepatitis of severity ranging from mild to fulminant (Kumar, 2013). Infection is transient in about 90% of adult and 10% of newborn, and persistent in the remainder. Most cases of acute hepatitis are subclinical, and less than 1% of symptomatic cases are fulminant (Robinson, 2010).

#### 2.1.7.1 Acute hepatitis B infection

Acute liver failure (ALF), earlier known as fulminant hepatitis, is a rare but dramatic clinical syndrome characterized by the sudden loss of hepatocytes, resulting in an indi vidual with no preexisting liver disease (Chen *et al*.,2019). After exposure to the virus, there is a long, asymptomatic incubation period, which may be followed by acute disease lasting many weeks to months. The

natural course of acute disease can be tracked using serum markers(Kumar,2013).

During the acute infection, hepatitis B does not appear to induce an intra-hepatic innate immune response and instead, it acts as a ‘stealth’ virus early in the infection (Spearman,2013).

Hepatitis B surface antigen (HBsAg) appears before the onset of symptoms, peaks during overt disease, and then declines to undetectable levels in 3 to 6 months (Barker and Murray,2010). Anti-HBs antibody does not rise until the acute diseases over and usually is not detectable for a few weeks to several months after the disappearance of HBsAg. It may persist for life, conferring immunity; this is the basis for current vaccination strategies using non-infectious. HBsAg, HBeAg, HBV-DNA, and DNA polymerase appear in serum soon after HBsAg signify active viral replication (Kumar,2013).

Persistence of HBeAg is an important indicator of continued viral replication, infectivity, and probable progression to chronic hepatitis. The appearance of anti-HBe antibodies implies that an acute infection has peaked and is on the wane (Kumar,2013).

**2.1.7.2 Chronic hepatitis B infection**

Chronic HBV is defined as persistence of serum HBsAg for more than 6 months (Yim, 2019).

Chronicity occurs rarely when the infection is contracted in adulthood, but is common in neonates and young children(Spearman ,2013). It is estimated that there are 240 million people who are chronically infected with HBV globally 15%–40% of infected patients will develop serious liver disease, resulting in up to 1.2 million deaths per year. HBV infection is the tenth leading cause of death worldwide (Burns and Alexander, 2014).

Chronically infected patients are unable to sustain an immune response to HBV and may experience intermittent episodes of hepatocyte destruction in

an attempt to clear virally infected hepatocytes, in what can be termed ‘flares’(Chisari and Ferrari,2012).

The complication rate of chronic hepatitis B is associated with the degree of viral replication, inflammation, and fibrosis. The risk for cirrhosis is also increased in the presence of fibrosis, long disease duration, male gender, comorbidities like alcohol consumption, diabetes mellitus type 2, obesity, and co-infection in particular with HDV or HIV (Niederau ,2016).

Age is also an important host factor determining the risk of chronicity. Following acute exposure to HBV, 90% of neonates born to HBeAg-positive mothers, 20 - 50% of infants and children under the age of 5 years, and <5% of adults will develop chronic hepatitis B infection. Viral variants may also influence the course and outcome of the disease. In addition, and only rarely and in the setting of profound immune suppression, the virus can be directly cytopathic (Spearman ,2013).

Most chronic HBV-infected patients have low viral replication with a lack of HBV e antigen (HBeAg) and absence of significant findings from liver biopsy. Generally, these subjects have good outcome and low risk of developing cirrhosis or liver cancer. They are called inactive carriers (Westin *et al*.,2019). On the other hand, there are patients who develop more severe histological lesions and have high HBV DNA levels. These subjects have high risk of developing HBV complications after years or decades of chronic infection (Burns and Alexander, 2014).

Other presentation of chronic HBV infection is the immune-tolerant phase, which is common among newborns of HBV female carriers. This situation is usually detected among children and teenagers. There is high HBV replication, HBeAg persistence, and high infectivity potential. Liver biopsy shows mild histological activity, and biochemical analysis shows aminotranferase levels under the reference range (Galizzi*et*,2010).

**Table (2. 1)** Phases of chronic hepatitis B infection (Chisari and Ferrari, 2012).

| | ALT | HBVDNA | HBeAg | Liver Histology |
|---|---|---|---|---|
| Immune-tolerant Phase | Normal | >1million IU/ml | Positive | Minimal inflammation and Fibrosis |
| HBeAg-Positive immune active phase | Elevated | ≥2000 IU/ml | Positive | Moderate-to-severe inflammation or fibrosis |
| Inactive CHB phase | Normal | < 2000 IU/ml | Negative | Minimal necroinflammation but variable fibrosis |
| HBeAg-Negative Reactivation phase | Elevated | ≥2000 IU/ml | Negative | Moderate-to-severe inflammation or fibrosis |

**2.1.7.3. Hepatocellular Carcinoma**

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and about 350 million people globally are chronically infected with HBV. Chronic HBV infection accounts for at least 50% cases of HCC worldwide (Xie, 2017). Risks of HCC among HBV-infected patients vary by several factors, the major one being serum HBV-DNA levels. Although there is no discrete cutoff level, having greater than $Log_{10}$ 5/mL viral copies confers a 2.5- to threefold greater risk over an 8- to 10-year follow-up period than does having a lower viral load. The cumulative incidence of HCC increases with serum HBV-DNA levels (Eun and Young, 2016). A recent hospital-based cohort study further validated the HCC risk, showing it started to increase when the HBV-DNA level was higher than 2000 IU/ml.In addition to HBV-DNA levels, the clinical

significance of quantitative hepatitis B surface antigen (HBsAg) has become increasingly recognized (Omata , 2017).

One particularly important intermediate aspect of a decades-long chronic HBV infection includes the development of HBV-associated cirrhosis prior to HCC development and it is generally accepted that the majority, potentially as much as 70– 90%, of all HCC occurs in the context of decompensated cirrhosis and a strong relationship exists between chronic HBV infection and cirrhosis (Lamontagne,2016). There are three reported mechanisms by which HBV promotes carcinogenesis: HBV proteins are involved in many signaling pathways in hepatocytes, thereby affecting the expression and functions of specific genes and contributing to live disorders and most of these changes are associated with HCC, integration of HBV DNA into the host genome alters the function of endogenous genes or induces chromosomal instability, and Inflammation-mediated alteration of specific signaling pathways contributes to tumorigenesis ( Xu, 2014). Chronic inflammation plays a vital role in the development of HCC and repeated cycles of inflammation-induced apoptosis and hepatocyte regeneration increase the risk of hepatocarcinogenesis (Eun and Young 2016).

### 2.1.8. Laboratory diagnosis

Serological markers for HBV infection consist of HBsAg, anti-HBs, HBeAg, antiHBe and anti-HBc IgM and IgG . The identification of serological markers allows : to identify patients with HBV infection ; to elucidate the natural course of chronic hepatitis B (CHB) ; to assess the clinical phases of infection ; and to monitor antiviral therapy ( CDC, 2012 ).

#### 2.1.8.1. Hepatitis B markers (Ags ) in blood

HBsAg is the serological hallmark of HBV infection. After an acute exposure to HBV, HBsAg appears in serum within 1 to 10 weeks . Persistence of this marker for more than 6 months implies chronic HBV infection (Eun and

Young 2016). Serum HBsAg titer are higher in patients with HBsAg-positive CHB than in HBeAg-negative CHB ( Jaroszewicz *et al*., 2010).

Monitoring of quantitative HBsAg levels predicts treatment response to interferon and disease progression in NBeAg-negative CHB patients with normal serum alanine aminotransferase levels (Martinot-Peigoux *et al*., 2013).

HBcAg is an interacellular presence in infected hepatocyte, thus it is not identified in the serum (Eun and Young, 2016).

HBeAg marker indicates viral replication and risk of transmission of infection, and seroconversion of HBeAg to anti-HBe is associated with remission of liver disease. However, some anti-HBe reactive subjects continue to have active viral replication and hepatic disease caused by mutations in the pre-core and core region in the HBV genome, which reduces the production of HBeAg (Villar ,2015).

**2.1.8.2. Hepatitis B markers (Abs) in blood**

Anti-HBs is known as a neutralizing antibody, and confer long-term immunity and in patients with acquired immunity through vaccination, anti-HBs is the only serological marker detected in serum. In the past HBV infection, it is present in concurrence with anti-HBc IgG (Song and Kim, 2016).

Occasionally, the simultaneous appearance of HBsAg and anti-HBs has been reported in patients with HBsAg positive and in most cases, anti-HBs antibodies are unable to neutralize the circulating viruses, thus these patients are regarded as carriers of HBV(Song and Kim ,2016).

The IgM anti-HBc is usually interpreted as a marker for early acute disease; however,in some patients, anti-HBc IgM levels can persist for up to 2 years after acute infection, and in patients with chronic active hepatitis, IgM antibody levels can rise during periods of exacerbation. An anti-HBc IgM titer is particularly helpful for screening blood donors,because this antibody is usually present during the window between HBsAg disappearance and anti-HBs appearance. The Immunoglobulin G antibodies directed against the core

antigen develop in the later phases of acute disease and usually persist for life (Frederick and Southwick, 2010).

**2.1.8.3. Polymerase chain reaction (PCR) and real time PCR tests**

PCR is a simple, yet elegant, enzymatic assay, which allows for the amplification of a specific DNA fragment from a complex pool of DNA (Caliendo *et al*., 2017).

PCR can be performed using source DNA from a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes. Only trace amounts of DNA are needed for PCR to generate enough copies to be analyzed using conventional laboratory methods. For this reason, PCR is a sensitive assay (Garibyan and Avashia,2013).

However, quantitation of HBV DNA in the serum provides an alternative to cccDNA detection, through less invasive method. According to the recommendations of the Taormina Group, detection of very low levels of HBV DNA should be done with highly sensitive PCR using primers specific for highly conserved sequences (genotype independent) of different HBV genomic regions. It has been observed that sensitivity of the HBV DNA detection by PCR may vary across different genetic regions of the HBV genome (Datta *et al*.,2014).

Most PCR based methods of HBV DNA detection for clinical purposes have a sensitivity of 50-200 IU/mL with dynamic range of 4-5 $\log_{10}$ IU/mL, in comparison, real-time PCR based assays have higher sensitivity (5-10 IU/mL) with a wider dynamic range 8-9 $\log_{10}$ IU/Ml (Aretzwelier *et al*., 2019).

Unlike traditional PCR, real-time PCR, with its increased accuracy, wider linear range, and reproducibility, is widely used for the quantitative detection of HBV DNA. Currently, most HBV DNA quantification reagents use one pair of primers and a single probe for a given HBV genotype test. If HBV genetic variations exist in these primer or probe regions, the actual viral load of HBV will be underestimated by the assay. Mutations in the probe region of

the COBAS Amplicor test caused by lamivudine led to the underestimation of the HBV DNA level of a chronic hepatitis patient (Liu,2017).

### 2.1.9. prevention

Prevention is far simpler than treatment, particularly in the case of HBV, which requires lifelong treatment in most cases. Besides avoiding transmission from infected people via blood supply screening and universal precautions, vaccination is the most important means of reducing the global burden of disease (Rajbhandari and Chung, 2016).

There are a number of settings in which post exposure prophylaxis in the form of passive immunization alone or in conjunction with hepatitis B vaccine is either necessary or desirable. Before the advent of vaccine, passive immunization with antiHBs was the sole option (Joshi, 2010).

Hepatitis B immunoglobulin (HBIG) is a purified solution of human immunoglobulin that could be administered to the mother, newborn, or both and it offers protection against HBV infection when administered to pregnant women who test positive for HBeAg or HBsAg, or both. When HBIG is administered to pregnant women, the antibodies passively diffuse across the placenta to the child to protect against HBV infection. This works best during the last third of pregnancy (Eke, 2017).

### 2.1.10. Treatment

Acute hepatitis B infection does not usually require treatment and most adults clear the infection spontaneously. Early antiviral treatment may be required in fewer than 1% of people, whose infection takes a very aggressive course (fulminant hepatitis) or who are immunocompromised (Hoofnagle *et al*., 2011).

On the other hand, treatment of chronic infection may be necessary,the aims of treatment of chronic hepatitis B are to achieve sustained suppression of HBV replication and remission of liver disease. the ultimate goal is to prevent cirrhosis, hepatic failure and HCC. Parameters used to assess treatment

response include normalization of serum ALT, decrease in serum HBV DNA level, loss of HBeAg with or without detection of anti-HBe, and improvement in liver histology (Locarnini,2013). At the 2000 and 2006 NIH conferences on Management of Hepatitis B, it was proposed that responses to antiviral therapy of chronic hepatitis B be categorized as biochemical (BR), virologic (VR), or histologic (HR), and as on-therapy or sustained off-therapy. Standardized definitions of primary nonresponse, breakthrough and relapse were also proposed. (Hoofnagle *et al*., 2011).

Currently, seven therapeutic agents have been approved for the treatment of adults with chronic hepatitis B in the United States,Lamivudine (Epivir), Adefovir (Hepsera), Tenofovir (Viread), Telbivudine (Tyzeka) and Entecavir (Baraclude), and the two immune system modulators interferon Alpha-2a and PEGylated Interferon Alpha- 2a (Pegasys) (Gish *et al*., 2015). The use of interferon, which requires injections daily or thrice weekly, has been substituted by long-acting PEGylated interferon, which is injected only once weekly (Albert and Caporaso, 2011).

### 2.1.11. Vaccination

Two single-antigen recombinant hepatitis B vaccines are commercially available, Recombivax HB® (Merck & Company, Inc.) and Engerix-B® (GlaxoSmithKline).

Recombivax HB contains 5–40 μg/mL of HBsAg protein, depending on the formulation, whereas Engerix-B contains 10–20 μg/mL. Both vaccines are licensed for persons of all ages. (CDC, 2017).

**Table (2. 2)** Recommended doses of currently licensed single-antigen hepatitis B vaccines (CDC,2017).

| Group | Recombivax HB | | Engerix-B | |
|---|---|---|---|---|
| | Dose (μg) | Volume(ml) | Dose (μg) | Volume(ml) |

| | | | | |
|---|---|---|---|---|
| Infants, children and adolescents younger than 20 years of age. | 5 | 0.5 | 10 | 0.5 |
| Adolescents 11–15 years (adult formulation administered on 2-dose schedule). | 10 | 1 | Not applicable | Not applicable |
| Adults 20 years of age or older . | 10 | 1 | 20 | 1 |
| Hemodialysis and other immunocompromise persons (adults 20 years or older) | 40 | 1 | 40 | 2 |

Twinrix® (GlaxoSmithKline), a combination of hepatitis A and B vaccine, is also available for use in persons 18 years of age and older. Twinrix consists of the antigenic components used in HAVRIX® (hepatitis A vaccine, Glaxo Smith Kline) and Engerix-B. In addition, Pediarix® (GlaxoSmithKline), another combination vaccine, contains recombinant HBsAg, diphtheria and tetanus toxoids and acellular pertussis adsorbed (DTaP), and inactivated poliovirus. However, these combination vaccines may not be administered to infants younger than 6 weeks of age; only singleantigen hepatitis B vaccine may be used for the birth dose. Administration of 4-dose hepatitis B vaccine schedules, including schedules with a birth dose followed by a combination vaccine series, is permissible (CDC, 2017).

**Table (2. 3)** Recommended doses of currently licensed combination hepatitis B vaccines (Lee *et al.,* 2019).

| Group | PEDIARIX | TWINRIX |
|---|---|---|

| | Dose (µg ) | Volume (ml) | Dose (µg ) | Volume (ml) |
|---|---|---|---|---|
| Infant (6 weeks and older) | 10 | 0.5 | NA | NA |
| Children (1–6 years) | 10 | 0.5 | NA | NA |
| Adolescents (11–17 years) | NA | NA | NA | NA |
| Adults (18 years or older) | NA | NA | 20 | 1.0 |

The vaccination schedule for infants includes 3 doses of vaccine in the first 18 months of life. The first dose should be given at birth with a minimum interval between doses 1 and 2 of 4 weeks, and a minimum interval of 8 weeks between doses 2 and 3. Dose 3 of hepatitis B vaccine should not be given before 24 weeks of age (168 days). The minimum interval between dose 1 and 3 is 16 weeks. Infants born to HBsAg-positive women or women with unknown HBsAg status should be immunized with the hepatitis B vaccine and HBIG within 12 hours of birth, regardless of birth weights.
(Lee *et al.,* 2019).

## 2.2.Cytokines

### 2.2.1. Roles and Nomenclature

Cytokines are soluble molecules plays an extremely important role in clinical immunology. They are act as stimulatory or inhibitory signals between cells. Cytokines that initiate chemotaxis of leucocytes are called chemokines (Horton-Szar *et al,* 2012).

It is Stimulate growth and differentiation of lymphocyte, activates immune cells to eliminate microbes & Ag, stimulate hematopoiesis and used in medicine as therapeutic agent (Abbas *et al.,* 2015). It nomenclature is according to producing cell is divided into: monokines produced by macrophage/monocyte, lymphokines produced by lymphocyte, Interleukins produced by leucocytes and act on other leucocytes eg IL-1& IL-2 & IL-3 and

biologic response modifier which used clinically to increase or reduce immunity. (Hawas,2016).

### 2.2.2. Properties of cytokines

Cytokines are produced transiently in response to antigen and usually acts on same cell that produces the cytokine (autocrine) or nearby cells (paracrine) and each cytokine has multiple biologic actions(Pleiotropism) Multiple cytokines may share the same or similar biologic activities (Redundancy) (Reche., 2019).

### 2.2.3. Cell produces cytokines

Cytokines are a cell-signaling group of low molecular weight extracellular polypeptides /glycoprotiens synthesized by different immune cells, mainly, by T cells, neutrophils and macrophages (Ma *et al*., 2018).

The production and release of cytokines from innate immune cells are critical responses to inflammation and infection in the body. Innate immune cells comprise populations of white blood cells such as circulating dendritic cells (DCs), neutrophils, natural killer (NK) cells, momocytes, eosinophils, and basophils, along with tissueresident mast cells and machrophages (Lwasaki and Medzhitov, 2010).

Residing at the frontline of defence in immunity, these cells control opportunistic invasion by a wide range of viral, fungal, bacterial and parasitic pathogens, in part by releasing a plethora of cytokines and chemokines to communicate with other cells and thereby to orchestrate immune response (Vazquez *et al*., 2015). This array of soluble mediators secreted by different innate immune cells includes TNF, IFNγ, interleukins

IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, CCL/4RANTES, and TGFβ (Lacy and Stow,2011).

Th1 subset secreted IL-2, IFNγ, and TNF, and is responsible for many classic cellmediated function including activation of cytotoxic T lymphocytes and macrophages ( Siransy *et al* ., 2018).

The main function is Cellular immune system Maximizes the killing efficacy of the macrophages and the proliferation of cytotoxic CD8+ T cells. Also promotes the production of IgG, an opsonizing antibody. Other functions IFNgamma increases the production of interleukin-12 by dendritic cells and macrophages, and via positive feedback, IL-12 stimulates the production of IFN-γ in helper T cells, thereby promoting the Th1 profile. IFN-gamma also inhibits the production of cytokines such as interleukin-4 (Christensen *et al*., 2018).

The Th2 subset secreted IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IL-25 .The main functions Humoral immune system Stimulates B-cells into proliferation, to induce Bcell antibody class switching, and to increase neutralizing antibody production (IgG, IgM and IgA as well as IgE antibodies (Owen *et al*., 2013). Also Interleukin-4 acts on helper T cells to promote the production of Th2 cytokines (including itself; it is auto-regulatory), while interleukin-10 (IL-10) inhibits a variety of cytokines including interleukin-2 and IFN-γ in helper T cells and IL-12 in dendritic cells and macrophages ( Christensen *et al*., 2018).

The Th17 are a subset of pro-inflammatory T helper cells involved in recruiting leukocyres and incducing inflammation (Hammerich, 2019).

The main effector cytokines are IL-17A, IL-17F, IL-21, and IL-22,and granulocyte-macrophage colonystimulating factor (GM-CSF). IL-17 family cytokines (IL-17A and IL-17F) target innate immune cells and epithelial cells, among others, to produce G-CSF and IL-8 (CXCL8), which leads to neutrophil production and recruitment (Zambrano and Zaragoza,2017).

The Th9 subsets secreted IL-9 which have a role in the induction and the pathogenesis of atopic disease, antiparasite immunity and immune pathological disease of the gut (Kaplan *et al*., 2015). Th9 cells have also shown both pro- and anti-tumorigenic activity, depending on the type of cancer (Tan *et al*., 2017).

### 2.2.4. Types of cytokines according to T helper

#### 2.2.4.1. Tumor necrosis factor (TNF)

Tumor necrosis factor (TNF, cachexin, or cachectin; once named as tumor necrosis factor alpha or TNFα) is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction (Victor and Gottlieb,2014) . It is produced chiefly by activated macrophages, although it can be produced by many other cell types such asCD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. TNF is a member of the TNF superfamily, consisting of various transmembrane proteins with a homologous TNF domain (Swardfager *et al*., 2010)

The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL1- & IL6-producing cells( Bobińska, 2017).

TNF-β, a type II transmembrane protein, is an important key in the development of lymph nodes and Peyer's patches, and also for the maintenance of secondary lymphoid organs. The expression of TNF-β is mainly stimulated by lymphocytes.

TNF-α will be better described in the following topics (Drutskaya *et al*., 2010). Although it were discovered many receptors along the decades, two are best known: TNFR1 (55 kD) and TNFR2 (75 kD). Both receptors are plasma membrane trimmers, while TNFR1 is expressed by most human cells and TNFR2 is mainly produced by immune system cells. It is important to mention that TNFR2 have a higher affinity to TNF. They are related to inflammatory reactions, so that a cytokine bind to the receptor, it induces the recruitment of proteins that are important for the process (Kalliolias and Ivashkiv, 2016) .

### 2.2.4.2. Interferons (IFN)

Interferons (IFN) are a group of signaling proteins made and released by host cells in response to the presence of several viruses. In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses (Levy *et al*.,2011).

Interferons are named for their ability to "interfere" with viral replication by protecting cells from virus infections. It is also have various other functions: they activate immune cells, such as natural killer cells and macrophages; they increase host defenses by up-regulating antigen presentation by virtue of increasing the expression of major histocompatibility complex (MHC) antigens( Espinosa *et al*., 2017).

### 2.2.4.3. Interleukins

Interleukins (ILs) are a group of secreted proteins with diverse structures and functions. These proteins bind to receptors and are involved in the communication between leukocytes. They are intimately related with activation and suppression of the immune system and cell division. The interleukins are synthesized mostly by helper CD4+ T lymphocytes, monocytes, macrophages and endothelial cells ( Seyfizadeh and Babaloo, 2015). ILs are grouped in families based on sequence homology and receptor chain similarities or functional properties (Catalan-Dibene *et al*., 2017).

#### 2.2.4.3.1. Interleukin-1 family:

Is a group of 11 cytokines that plays a central role in the regulation of immune and inflammatory responses to infections or sterile insults ( Dinarello , (2015). 7 ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), 3 receptor antagonists (IL-1Ra, IL-36Ra and IL-38) and 1 anti-inflammatory cytokine (IL-37) ( Garlanda *et al*., 2013).

#### 2.2.4.3.2. Interleukin-2 family

The IL-2 cytokine family, also known as the common γ-chain family, is composed by ILs 2, 4, 7, 9, 15 and 21. All these ILs bind to the common γc

receptor, also called CD132. These cytokines act as growth and proliferation factors for progenitors and mature cells (Sim and Radvanyi, 2014).

### 2.2.5. Interleukin-12 (IL-12)

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ) (Frydas *et al.*, 2012).

Naturally produced by dendritic cells, macrophages, neutrophils, and human Blymphoblastoid cells in response to antigenic stimulation. IL-12 is composed of a bundle of four alpha helices. It is a heterodimeric cytokine encoded by two separate genes, IL-12A (p35) and IL-12B (p40). The active heterodimer (referred to as 'p70'), and a homodimer of p40 are formed following protein synthesis (Veerdonk *et al*.,2011).

IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T cells. It stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factoralpha (TNF-α) from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ. T cells that produce IL-12 have a coreceptor, CD30, which is associated with IL-12 activity (Hsieh *et al*.,2015).

IL-12 plays an important role in the activities of natural killer cells and T lymphocytes. It mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes (Vignali *et al*., 2012) . There also seems to be a link between IL-2 and the signal transduction of IL-12 in NK cells. IL2 stimulates the expression of two IL-12 receptors, IL-12R-β1 and IL-12R-β2, maintaining the expression of a critical protein involved in IL-12 signaling in NK cells. Enhanced functional response is demonstrated by IFN-γ production and killing of target cells (Wang *et al*.,2015).

It has also anti-angiogenic activity, which means it can block the formation of new blood vessels. It does this by increasing production of interferon gamma,

which in turn increases the production of a chemokine called inducible protein-10 (IP-10 or CXCL10). IP-10 then mediates this anti-angiogenic effect. Because of its ability to induce immune responses and its anti-angiogenic activity, there has been an interest in testing IL-12 as a possible anti-cancer drug (Zheng *et al*., 2016).

IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by IL-12Rβ1 and IL-12Rβ2. IL-12Rβ2 is considered to play a key role in IL-12 function. Since it is found on activated T cells and is stimulated by cytokines that promote Th1 cells development and inhibited by those that promote Th2 cells development. Upon binding, IL-12R-β2 becomes tyrosine phosphorylated and provides binding sites for kinases, Tyk2 and Jak2. These are important in activating critical transcription factor proteins such as STAT4 that are implicated in IL-12 signaling in T cells and NK cells. This pathway is known as the JAK-STAT pathway (Hsieh *et al*.,2015).

### 2.2.6. IL-12 and Hepatitis B Virus

The host response to viruses relies on a complex interaction of several cell systems; the cells of the innate immune system, the dendritic cells, which are key in priming and directing the virus-specific T-cell response; and the T cells, which are the main antiviral effectors. After infection of the hepatocyte various cellular and humoral responses have been postulated that are aimed at elimination of the virus (Wang *et al*.,2015).

Antibody The earliest responses are non-specific and include the interferon system, natural killer cells, and non-specific activation of Kupffer cells. The precise role of several of these unspecific mechanisms is not well understood in HBV infection although it was recently shown that natural killer T-cell activation inhibits HBV replication in vivo. after these non-specific responses, immune responses directed specifically against viral proteins become

important. The two major arms of the immune system are the humoral arm, which consists of B lymphocytes that produce, and the cellular arm, which is composed of various cell types, including macrophages and T lymphocytes virus (Li *et al*.,2015).

Recent studies have defined many T helper cell subsets Th1 and Th2, which are characterized by distinct and mutually exclusive patterns of cytokine production and different functions. Th1 cells produce interferon (IFN)-γ and interleukin (IL-12) and promote cellular immune reactions while Th2 cells produce IL-4, IL-5, IL10 and enhance humoral immune response. Th1/Th2 imbalances are important in the pathogenesis of chronic HBV infections in humans. (Jiang *et al*., 2010).

IL-12 has a central role in mounting an effective cellular immune response directed toward the elimination of intracellular pathogens and it is known that the production of this cytokine is important for viral clearance. (Meher *et al*.,2010).

**2.2.7. Previous study**

In Iraq 0sama and his colleagues quantified serum IL-12 in patients with HBV and healthy controls, Serum level of IL-12 showed significant difference in patients with HBV compared to controls (Osama and Alsaffar, 2019).

Meher and his colleagues in India was determined serum IL-12 level in One hundred and fifty-seven patients with hepatitis B (34 acute and 123 chronic HBV cases) and thirty control groups. In healthy controls, the mean level of IL-12 was 39.14 pg/ml. In patients with AHB and CHB, these were 49.56 and 34.88 pg/ml respectively. This suggests that IL-12 appears to play a preeminent role in prognosis of the disease (Meher *et al*., 2010).

Study was conducted in London. A total of 98 patients with CHB and 4 healthy volunteers participated in the study. PBMC from CHB patients were stimulated with HBV derived peptides and cultured for 10 days in the presence or absence of IL-12 or IFN-α. We observed that IL-12 was able to consistently

increase the amount of IFN-γ produced per cell, indicated by an increase in IFN-γ mean fluorescence intensity (MFI) in CD8 T cells responding to HBV peptide stimulation (Anna *et al*., 2013) .

In Cairo 2015 Abd El-Gawad Fahmi and his colleagues measured serum levels of IL12 in patients of different groups . The liver function pattern of these groups were monitored. Data Recorded revealed that there was a highly significant negative correlation between Interleukin-12 and ALT, AST, Albumin, Total Bilirubin and Total Bilirubin ($P < 0.05$). This suggests that IL-12 can be used as a biomarker for the hepatic infection (Abd El-Gawad *et al*., 2015).

Other study was conducted in Poland 2010. CD4 T cells were isolated from peripheral blood of 20 children with chronic active hepatitis B, cultured for 48h in presence of rHBcAg and of co-stimulator, IL-12 or in the absence (control). The most pronounced stimulatory effect was observed in the presence of IL-12 and resulted in peak levels of IFN-gamma production. The obtained results allowed concluding that the anti-HBV activity of Th1 lymphocytes is strongly induced by IL-12 and may contribute to viral clearance in children with chronic hepatitis B infection (Jacek *et al*,2010).

Study was conducted in Iran 2015, The purpose of this study was to investigate the possible association between expression levels of IL-12 gene with HBV infection in patients with HBV infection. 30 HBV patients and 30 healthy controls. The results of study demonstrated that the difference in mean of IL-12 gene expression between healthy subjects and HBV patients is statistically significant (Abdalhossein and Ahmad, 2015).

Other study was conducted in London to investigating the immunoregulatory role of IL-12 in 72 chronic hepatitis B virus (HBV) carriers. Chronic HBV carriers had higher serum levels of IL-12 and IL-12 p40 in comparison with controls ($P < 0.01$), suggesting that IL-12 production is not impaired ( Rossol *et al*., 2010).

In China Yang and his colleagues quantified serum IL-12 by ELISA in 213 patients with CHB and 20 healthy controls, levels of IL-17 proteins in CHB patients were higher than those in the healthy control group($p<0.05$) (Yang *et al*., 2018).

In Germany Three hundred thirty-three patients with HBV infection, not undergoing antiviral therapy, were included in cross-sectional study, Significant differences were documented in the level of IL-12 between the patients and control (steffen *et al*., 2019).

Study was conducted in Japan. IL-12 is evaluated in 12 patients with chronic, IL12 in CHB is significantly higher ($p<0.05$) than in controls (Kishida, 2019).

In China 2015. The aims of this study were to detect the expression of IL-12 in CHB patients and explore the molecular mechanism of HBV-induced IL-12 expression. The results showed that serum levels and hepatic expression of IL-12 were significantly upregulated in CHB patients (Hong *et al* ., 2015).

# Chapter Three

## Materials and Methods

# CHAPTER III

# MATERIALS AND METHODS

## 3.1. Study design

This was prospective case control study.

## 3.2. Study area and duration

Study was conducted in Saba Laboratory (hepatitis B virus infections center) in Khartoum state during the period from April to February (2020).

## 3.3. Study population

Study population consisted of 80 Sudanese individuals of age between 17-65 years, divided into 2 groups as follows-HBV infected made up of 40 patients and 40 apparently control group.

## 3.4. Inclusion criteria

Patients were considered in chronic HBV infected control group was age and sex matched apparently health subjects.

## 3.5. Exclusion criteria

All HBV infected patients with situation that affected cytokine level including physiological factors such as (Pregnancy, smoking and alcohol consumption) and others diseases such as (autoimmune diseases, infectious diseases, allergy, hypersensitivity, cancer, heart failure and Parkinson diseases).

## 3.6. Sample size

A total of 80 subjects were enrolled in this study. 40 samples were collected from HBV infected patients and 40 samples were collected from healthy volunteer.

## 3.7. Data collection

Samples were collected randomly, Questionnaire was used to collect demographic, clinical and laboratory data.

### 3.8. Ethical consideration

Permission to carry out the study was obtained from scientific and Ethical Committee, College of Medical Laboratory Science, Sudan University of Science and Technology . Also taken from laboratory manger. Every sample was collected after verbal approval by patients and volunteer.

### 3.9. Sampling

Three ml of venous blood was collected from patients and control in a Ethylenedi- aminetetraacetic acid container. Then the samples were centrifuged and serum was separated in a sterile container and stored at -20 ºC until analysis. plasma level of IL-12 were measured using ELISA. (Biolegend's ELISA MAXTM).

### 3.10. Principle and procedures

#### 3.10.1. Principle of the ELISA

Biolegend's ELISA MAXTM Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-12 specific monoclonal antibody is irst coated on a 96-well plate. Standards and samples are added to the wells, and IL-12 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-12 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish preoxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-12 present in sample. Finally, the stop Solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate reader. (www.biolegend,com).

##### 3.10.1.1. ELISA Procedure

**Day I** 100 µL of diluted capture antibody solution was added to each well, seal the plate and incubate overnight between 20˚C and 80˚C .

**Day II** plate washed 4 times with at least 300µL of wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper. To block the plate by adding 200 µL 1X Assay Diluents A to each well, seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly. Plate washed 4 times; add 100 µL Diluted standareds and samples to the appropriate wells. seal the plate and incubate at room temperature for 2 hours with shaking. then wash plate 4 times; add 100µL diluted detection antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking. wash plate 4 times; add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking. wash plate 5 times; soaking for 30 seconds to 1 minute per wash. add 100µL of freshly mixed TMB substrate solution to each well and incubate in the dark for 30 minutes. Add 100µL stop solution to each well. read absorbance at 450 nm and 570 nm within 15 minutes.

The absorbance 570 nmm can be substracted from the absorbance at 450 nm. (www.biolegend,com.)

### 3.10.1.2. ELISA Washer principle

First the wash solution is pump from the wash bottle, the solution is dispense to the cuvette by short pins, and then the wash liquid is aspirate from the cuvette by long pins, at the end the waste liquid was pumped into the waste bottle by the vacuum pupp.(www.diasource.be, 2020).

### 3.10.1.3. ELISA reader principle

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted. It is filtered by interference filters and focused onto the photodiodes. The photodiode converts the received light into an electrical signal which is transformed into a digital form, from which the

microprocessor calculates the absorbance, taking in account of the blank and dichromatic selection.(www.diasource.be ,2020).

### 3.11. Statistical analysis

Data was analyzed by using statistical package for social science {SPSS} (version 20) using Mean ± SD One-way ANOVA test and independent T test for testing difference significance, correlation test to find out correlation and frequencies to obtain mean and stander deviation. Probability value (*PV*) less than or equal 0.05 was considered statistically significant

# Chapter Four

## Results

# CHAPTER IV

# RESULTS

## 4.1. The Results

Eighty volunteers with varies age from 17-65 years were enrolled in this study,40 subjects are chronic hepatitis B virus patients,20/40 of them, (50%) were males and 20/40 (50 %) are females with mean age 30.2±12.3 years. Other 40 subjects are apparently healthy control,20/40 (50%) are males and 20/40 (50 %) are females with mean age 34.3±12.2 years .

Mean level of IL-12 in hepatitis B virus patient (693.94± 898.9 pg/ml), in control group (181.18±124.3 pg/ml) with statistically significant correlation between case and control (*p-value* 0.001) (Table 4-1).

Mean level of IL-12 was lower in males (259.4±561.6 pg/ml) than females (615.7±760.1), and showed statistically significant correlation (*P-value* 0.02) (Table 4-2).

Regarded to treatment there was statistical significantly correlate between patients using treatment compared to patient not use treatment (424.8±347.6 and 1097.7±1275 respectively) (*p. value* 0.05) (Table 4-3).

While showed no statistical significant correlate in IL-12 level between patients with symptoms of jaundice (834±955.5pg/ml) and patients have no symptoms of jaundice (523±818.3pg/ml) (*p. value* 0.282) (Table 4-3).

In blood transfused patients IL-12 level was decreased in patients received blood transfusion

(340.6±294.9pg/ml) when compared to patients didn’t receive blood transfusion (828±1013.3pg/ml) and this difference was statistically significant (*p-value* 0.025) (Table 4-3).

In vaccinated volunteers IL-12 level was decreased in patients how vaccinated (430.5±349.2pg/ml) when compared to patients didn’t (782±904.5pg/ml) and this difference was statistically significant (*p-value* 0.036). (Table 4-3).

The results showed IL-12 level did not affected with age group among case subject and *p.value* showed insignificant correlation (*P-value* 0.5) (Table 4-4).

**Tables (4. 1)** Comparison of IL-12 Level and Age between Hepatitis B Patients Group and Healthy Control Group.

| **Parameter** | **patients** | **Healthy control** | ***P-value*** |
|---|---|---|---|
| Age | 30.2±12.3 | 34.33±12.2 | 0.138 |
| IL-12 | 693.9±898.9 | 181.2±124.3 | 0.001 |

*****$p$-*value* $\leq 0.05$ = significant

**Tables (4. 2)** Comparison between IL-12 level among male and female subjects.

| **Sex** | **Mean ±STD** | ***p-value*** |
|---|---|---|
| Male | 259.4±561.6 | 0.02 |
| Female | 615.7±760.1 | |

**Tables (4. 3)** Comparison between IL-12 and Different Age Group.

| **Age groups (years)** | **IL12 level Mean ± STD** | ***p-value*** |
|---|---|---|
| **≤20** | 320.6±253 | 0.5 |
| **20-44** | 504.5±831.3 | |
| **≥45** | 437.6±687.8 | |

**Tables (4. 4)** Mean between level of Hepatitis B Virus Patients and Possible risk factors.

| **Variable** | | **Mean ±STD** | ***P-value*** |
|---|---|---|---|
| Treatment | Yes | 424.8±347.6 | 0.05 |
| | No | 1097.7±1275 | |
| Blood transfusion | Yes | 340.6±294.9 | 0.025 |
| | No | 828±1013.3 | |
| Jaundice | Yes | 834±955.5 | 0.282 |
| | No | 523±818.3 | |
| Vaccine | Yes | 430.5±349.2 | 0.036 |
| | No | 782±904.5 | |

# Chapter Five
# Discussion, Conclusions and Recommendations

# CHAPTER V

# DISCUSSIONS, CONCOLUSIONS AND RECOMMENDATIONS

## 5.1. Discussion

Interleukin_12 is an important immunoregulatory cytokine that is produced mainly by antigen-presenting cells. IL-12 induces interferon-γ (IFN-γ) production and triggers CD4+ T cells to differentiate into type 1 T helper (Th1) cells. Many studies have suggested that IL-12 could play a vital role in treating many diseases, such as viral and bacterial infections and cancer. **(**Ippolito *et al*.,2010).

In the present study plasma level of IL-12(pg/ml) is significantly higher in hepatitis B virus patients compared to healthy control group (*p value* 0.001), This finding was supported by Idress who demonstrate that significantly elevated of serum level of IL12 protein in CHB patients compared to healthy control (*p-value* <0.05) ( Idress , 2019),Gish and his colleague showed that significant increases of IL-12 in CHB patients compare(d with healthy control group (*P-value* <0.05) (Gish *et al*., 2015).

The study revealed that statistical correlation between IL-12 and sex in hepatitis B virus patients and healthy control group (*p-value* 0.02). This results was supported by Owen and his colleague demonstrate significant difference between sex and IL-12 (Owen *et al*., 2013), olso Meher who demonstrate that no statistical difference between sex and IL-12 (P-value 0.004) this due to use few number of sample (Meher *et at*., 2010).

Our result also demonstrate that significant statistical difference in IL-12 level between who received blood transfusion and didn't receive (*P-value* 0.025) this may be due to blood transfusion contains therapy or antibodies reduce IL12 level , Chisari and Ferari who demonstrate that they was significant statistically difference between blood transfusion and IL-12(*P.value* 0.012) (Chisari and Ferari , 2012).

Also the study demonstrate that no statistical difference between IL-12 and Jaundice (*P-value* 0.282) and statistical difference between IL-12 and treatment (*P-value* 0.05) ,also statistical difference between IL-12 and vaccine (*P-value* 0.036 ) this result may be due to vaccine components reduction IL12 level.

The results showed IL-12 level did not affected with age group among case subject and *p.value* showed insignificant correlation (*P-value* 0.5) on the contrary Zheng and his colleague showed that IL-12 level increasing 34 trend, correlated with the age (Zheng *et al*., 2016) may be this difference due to levels of exposure to potential risks environment and sample size. Study revealed that no statistical correlation between IL-12 levels and age in hepatitis B virus patients group and healthy control group (*P-value* 0.138).

## 5.2 Conclusion

- Plasma level of IL-12 was compared in hepatitis B patients group and healthy subjects group with statistically significant difference *(P-value 0.001*).
- There was significant statistical difference between IL-12 level with gender.
- There was insignificant statistical difference between IL-12 level with age.

## 5.3 Recommendations

**Further studies may be conducted considering:**

- The increase of sample size and patient's population (chronic carriers, patients on antiviral therapy, patient has not starting antiviral treatment yet, patient with liver cirrhosis and hepatocellular carcinoma) is likely to enhance our understanding of IL-12 roles and biological activities.
- Regular measurement of IL-12 level in Sudanese hepatitis B patients.
- Estimating the histological activity indices and fibrosis score in liver biopsies, and ultrasound finding will yield great information about liver status and the effect of IL-12 during the different disease phases.
- Use the molecular techniqus
- Finally the IL12 can be considered usefulas prognostic marker for hepatitis B virus infection.

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# Appendcies

## Appendix (1)

**Sudan university of sciences and technology**

**Evaluation of Interleukin 12 among Sudanese Hepatitis B patients** Date / 2020

ID Number:……..

Age:……Years

Gender:

Male ( ) Female ( )

Duration of disease:

Less than six months ( ) More than six months ( )

Treatment:

Yes ( ) No ( )

Blood transfusion:

Yes ( ) No ( )

Jaundice:

Yes ( ) No ( )

Any chronic disease:

Yes ( ) No ( )

## Appendix(2)


**Elisa Kits**

# Appendix (3)

## Human IL-12/IL-23 (p40) ELISA MAX™ Deluxe Set

### Certificate of Analysis

**Product Name:** Human IL-12/IL-23 (p40) ELISA MAX™ Deluxe Set

**Product Cat. No:** 430704 (5 plates) / 430705 (10 plates) / 430706 (20 plates)

**Lot No:** B235741

**Expiration Date:** 31-MAR-2019

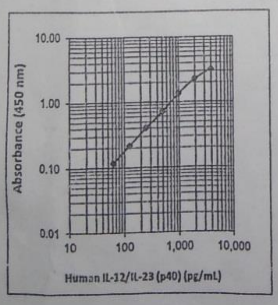
| Contents Description | Quantity (5 plates) | Volume (per bottle) | Part No. | Lot No. |
|---|---|---|---|---|
| Human IL-12/IL-23 (p40) ELISA MAX™ Capture Antibody (200X) | 1 vial | 300 µL | 79032 | B234995 |
| Human IL-12/IL-23 (p40) ELISA MAX™ Detection Antibody (200X) | 1 vial | 300 µL | 79033 | B234996 |
| Human IL-12/IL-23 (p40) Standard | 2 vials | 15 ng | 79034 | B226549 |
| Avidin-HRP (1,000X) | 1 vial | 60 µL | 79004 | B233033 |
| Substrate Solution A | 1 bottle | 30 mL | 78570 | B235092 |
| Substrate Solution B | 1 bottle | 30 mL | 78571 | B235093 |
| Coating Buffer A (5X) | 1 bottle | 30 mL | 79008 | B232549 |
| Assay Diluent A (5X) | 1 bottle | 60 mL | 78888 | B233503 |
| Nunc™ MaxiSorp™ ELISA Plates, Uncoated | 5 plates | - | 423501 | - |

**Storage Conditions**

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   - 2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   - 2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

*Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.*

Lot #: B235741




This standard curve is for demonstrative purposes only.
A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 4/13/17

BioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

FOR RESEARCH USE ONLY

BioLegend | 9727 Pacific Heights Blvd | San Diego, CA 92121 U.S.A.

### ELISA MAX™ Deluxe Set Protocol

**Materials to be Provided by the End-User**

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g $Na_2HPO_4$, 0.2 g $KH_2PO_4$, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N $H_2SO_4$.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

**Reagent Preparation**

| Reagents Description | Dilute with | Dilution for 1 plate |
|---|---|---|
| Coating Buffer A (5X) | Deionized Water | 2.4 mL in 9.6 mL DI $H_2O$ |
| Capture Antibody (200X) | 1X Coating Buffer A | 60 µL in 12 mL Buffer |
| Assay Diluent A (5X) | PBS | 12 mL in 48 mL PBS |
| Detection Antibody (200X) | 1X Assay Diluent A | 60 µL in 12 mL Buffer |
| Avidin-HRP (1,000X) | 1X Assay Diluent A | 12 µL in 12 mL Buffer |

**Standard reconstitution:** Reconstitute the lyophilized Human IL-12/IL-23 (p40) standard by adding 0.2 mL of 1X Assay Diluent A to make the 75 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 4,000 pg/mL by adding 53.3 µL of reconstituted standard stock solution to 946.7 µL 1X Assay Diluent A. Perform six two-fold serial dilutions of the 4,000 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

**Samples:** For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

**TMB Substrate Solution Preparation:** TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

**ELISA Procedure Summary**

**Day 1**

Add 100 µL diluted Capture Antibody solution to each well, seal the plat and incubate overnight between 2°C and 8°C.

**Day 2**

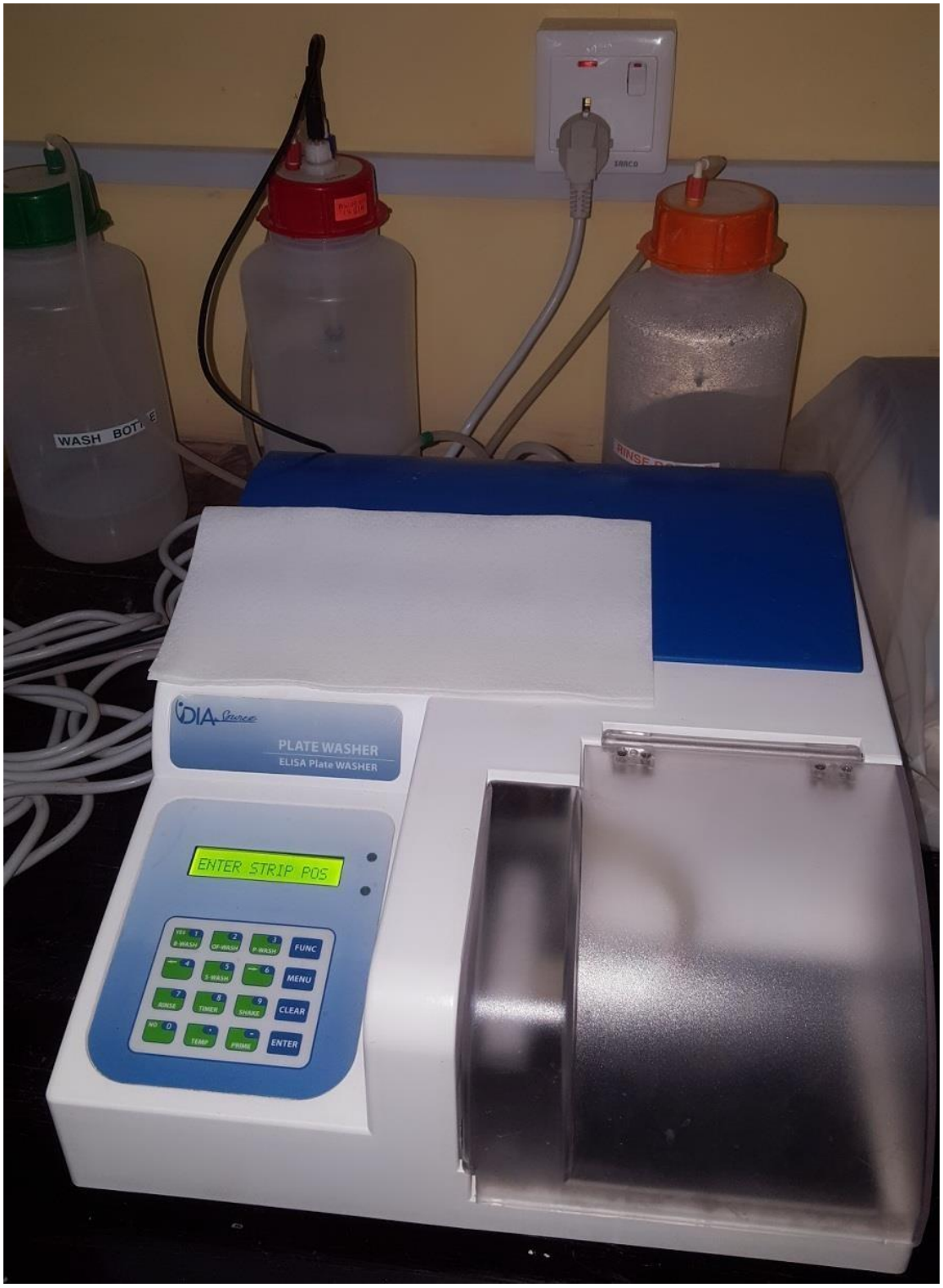
1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal plate and incubate at room temperature for 1 hour wit shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shakir
4. Wash plate 4 times* , add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each we seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incuba in the dark for 15 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracte from the absorbance at 450 nm.

*Plate Washing: Wash step is crucial to assay precision. Wash the plate with least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tap ping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at: www.biolegend.com/media_assets/pro_detail/datasheets/430704.pdf

BioLegend®

## Appendix (4)




**ELISA Washer**

## Appendix (5)

**ELISA micro plate**

## Appendix (6)


**ELISA Reader**